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PRINCIPAL INVESTIGATOR: Gang-Yi Wu, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030-3498

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Gang-Yi Wu, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030-3498

E-Mail: Gangyiw@bcm.tmc.edu

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13. ABSTRACT (Maximum 200 Words)
Neurofibromatosis type 1 (NF1) is a common dominant genetic disorder characterized by multiple benign and malignant tumors of neural origin and, often, cognitive deficits in children. The protein encoded by NF1, neurofibromin, contains a GAP domain, known to inhibit Ras-mediated signal transduction, a pathway known to be required for memory formation. This proposal will define the newly identified NF1-Syndecan2-CASK signaling complex in the regulation of synaptic Ras-MAPK activity and dendritic spine maturation. The specific Aims are: 1) To determine whether the NF1-syndecan-CASK signaling complex is an essential negative regulator for synaptic Ras-MAP kinase activity during synaptic maturation. 2) To define the role of the NF1-syndecan-CASK signaling complex in the formation and maturation of dendritic spines. 3) To assess if NF1-deficient cells have an altered capacity to undergo morphological plasticity after spaced depolarizing stimuli, and whether the deficits in morphology can be rescued by manipulating Ras-MAPK signaling. We have made excellent progress on developing several siRNAs and dominant negative constructs for NF1 GAP activity to specifically knockdown or inhibit NF1, and have begun to assess their effects on Ras-MAPK signaling and spine maturation. Furthermore, we have obtained compelling evidence showing that Nf1+/- neurons display hyperactive basal and evoked MAPK activity.

14. SUBJECT TERMS
NF1, syndecan2, cask, ras-MAPK signaling, dendritic spines, immunocytochemistry, fret, plasticity, EPHB2, NF1 knockout mice, dentate explant cultures, timelapse-imaging, confocal microscopy, RNA interefence, dominant negative mutants, knockout

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I. Introduction:

Neurofibromatosis type 1 (NF1) is one of the most common dominant genetic disorders characterized by multiple benign and malignant tumors of neural origin. About 50% of NF1 children also exhibit cognitive deficits such as spatial learning defects and reading difficulty. How mutations in a single gene lead to severe learning deficits is largely unknown. The protein encoded by NF1, neurofibromin, contains a GAP domain, known to inhibit Ras-mediated signal transduction. A recent report from Silva's group demonstrated that the learning deficits of heterozygous null mutant (Nf1+/-) mice could be rescued by genetic and pharmacological manipulations that decrease Ras function (Costa et al., 2002), suggesting that a tightly regulated Ras activity is critical for its function in synaptic plasticity. NF1 forms a tripartite complex with CASK, a synaptic PDZ protein, and Syndecan 2, a heparan sulfate proteoglycan (HSPG) (Hsueh et al., 2001). CASK has previously been proposed to function as multidomain scaffolding protein that organizes specific signaling complexes at contact sites, and may have a role in receptor localization. HSPGs are believed to function as co-receptors in many receptor tyrosine kinase signaling pathways, and Syndecan 2 is known to promote dendritic spine maturation. Therefore, in principle, this protein complex can function in both organizing the synaptic protein complex and mediating key signal transduction events during synaptogenesis and synaptic plasticity. The Objective of this study is to combine structural and functional analyses in conjunction with the assessment of the underlying signal transduction mechanisms at single cell level, to better understand the precise NF1 function in neurons and how dysregulation of this function leads to cognitive deficits in NF1 patients.

II. Body:

We propose to use multidisciplinary approaches, including time-lapse imaging confocal microscopy, molecular imaging with FRET, quantitative immunocytochemistry, and genetic mouse models as well as pharmacological and molecular manipulations such as dominant negative constructs and small interfering RNAs (siRNAs), to define the NF1 function in synapse formation and morphogenesis of dendritic spines. The three major tasks of this study are:

Task1: To determine if the NF1-syndecan-CASK signaling complex is an upstream regulator of the synaptic Ras-MAP kinase pathway

Task2: To assess the role of the NF1-syndecan-CASK signaling complex in regulation of dendritic spine morphology

Task3: To determine if NF1-deficient cells or NF1 deficient mice have an altered capacity to undergo morphological plasticity after spaced depolarizing stimuli, and if deficits in morphology can be rescued by manipulating Ras-MAPK signaling.

During the past funding period, we have made excellent progress on task 1 & 2. These results are in agreement with our hypothesis that the NF1-syndecan-CASK signaling complex plays an essential role in dendritic spine formation and plasticity, and it does so through its crucial role as a negative regulator for Ras (and MAPK) signaling. In the following sections, I will outline our detailed findings.

1). Task1: To determine if NF1-syndecan-CASK signaling complex is an upstream regulator of the synaptic Ras-MAP kinase pathway

Although the learning deficits of Nf1+/- mice can be rescued by genetic and pharmacological manipulations that decrease Ras function (Costa et al., 2002), it is somewhat surprising that there has been no evidence reported so far that the Ras-MAPK pathway is indeed altered in Nf1+/- neurons. To address this important issue, we used both immunoblot analysis and immunohistochemistry to assay the basal and depolarization-induced MAPK activity in wild type and Nf1+/- neurons (Figs.1 & 2). The results clearly demonstrate that as seen in many other types of cells, Nf1+/- neurons display hyperactive basal and evoked MAPK activity as compared to wild type neurons, suggesting that NF1 plays a critical role in the regulation of basal and depolarization-induced MAPK activity in neurons.

Small interfering RNAs (siRNAs, 21- to 22-Nucleotide) have recently emerged as a powerful tool to suppress gene expression through a process known as RNA interference or RNAi. We have put significant efforts into making this powerful tool for our use. Dr. Ming-Xiang Zhang has tested several available RNAi systems (including psilencer3.1 with H1 or U6 promoter (from Ambion); pSuperRNAi and the latest version EGFPpSuperRNAi (from Oligoengine)). Based on our experience, the pSuperRNAi system works best in neurons. We have constructed more than 10 different pSuperRNAi constructs for NF1. Western blot data showed that about half of them specifically reduced the NF1 protein level to an extent greater than 50% (Figs. 4 & 5). Non-related siRNA constructs or an siRNA for EGFP had no effect on NF1 expression at 48 hrs after transfection.

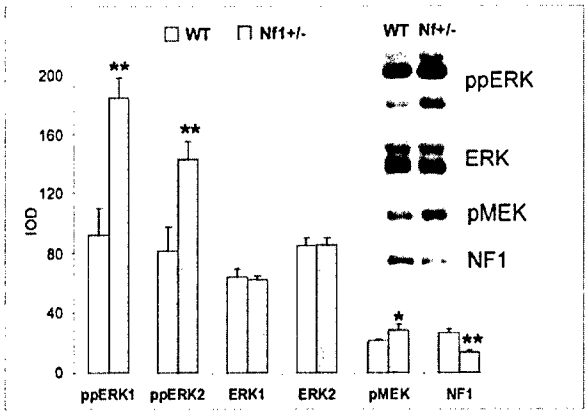

Fig.1 Hyperactive basal pMAPK activity in Nf1+/- hippocampal homogenates. Hippocampi were dissected from young adult WT and Nf1 +/- brains (n=6), homogenized in hypotonic lysis buffer, and resolved by SDS-PAGE. Western blots were probed for dually-phosphorylated ERK1 (ppERK1) and ERK2 (ppERK2), total ERK1 and -2, phospho-MEK, and NF1 protein. Densitometric values were mean +/- SD. NF1 antibody was from Santa Cruz; all others were from Cell Signaling. *p<0.05; **p<0.01 by Student's t-test.

To verify the specificity of our siRNAs for NF1, and further determine whether knockdown of NF1 also alter MAPK signaling as seen in Nf1+/- neurons, we have focused on two of the siRNAs: $siRNA_{INF1\text{-}i}$ and $siRNAI_{NF1\text{-}K}$ that target regions in the NF1GRDI or outside of the NF1GRDI, respectively. As shown in Figs.4 & 5, both constructs reduced the level of NF1 more than 50% when tested on Hela cell line, and as expected, pMAPK activity was significantly increased in cells expressing $siRNA_{INF1\text{-}i}$ or $siRNAI_{NF1\text{-}K}$. Remarkably, overexpression of NF1GRDI, a central domain of NF1 containing ~360 residues responsible for its Ras GAP activity, was able to largely rescue the pMAPK level in cells co-expressing the $siRNAI_{NF1\text{-}K}$ with NF1GRDI. Cells co-expressing the $siRNA_{INF1\text{-}i}$ with NF1GRDI still showed elevated hyperactive pMAPK and the slightly reduced pMAPK activity was due to incomplete knockdown of NF1GRDI by $siRNA_{INF1\text{-}i}$. A scramble negative control (with the same nucleotide composition as the siRNA but which lacks significant sequence homology to the genome) and a mismatched siRNA control (one or two bases in the middle of the siRNA sequence are modified to make them non-complementary to the target mRNA) are being developed to further validate the specificity of the gene knockdown results.

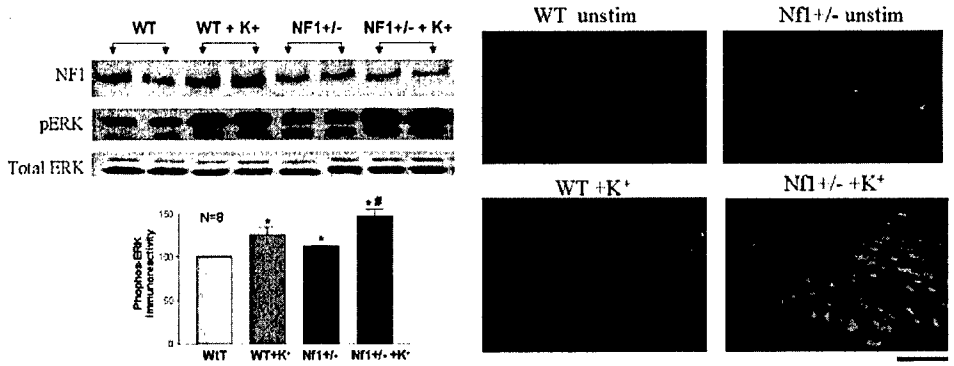

Fig.2 Hyperactive basal and high K+-induced pMAPK activity in acute Nf1+/- hippocampal slices. Hippocampi were dissected from young adult WT and Nf1 +/- brains (n=8), cut into 400μm slices, then stimulated by 90mM K+ for 3min. After 10min wash, the slices were either homogenized in hypotonic lysis buffer and assayed for immunoblots, or immunostained for pERK, total ERK, and NF1 protein. Densitometric values were mean +/- SD. *<0.05 compared to WT; #<0.05 compared to WT+K+ by Student's t-test.

As an independent approach, we have made use of dominant negative constructs for NF1 GAP. Two arginine residues of NF1 in the catalytic arginine loop (Arg^{1276} and Arg^{1391}) in NF1-GRD, whose alterations have been found in NF1 patients with severe phenotypes, are known to be important for catalysis of NF1-GAP activity

(Scheffzek et al., 1998). Mutational analysis of NF1-GRD revealed that replacing Arg^{1276} with lysine (R1276K) increases its binding affinity for Ras 1.85-fold but greatly reduces it GAP activity. The affinity of the double mutant R1276A/R1391K for the Ras-GTP form was slightly increased by 1.08 fold, and the GAP activity was decreased to 1/1080 compared to wild-type NF1-GRD (Ahmadian et al., 1997; Scheffzek et al., 1998). Therefore, these mutants are thought to act as dominant negative inhibitors for endogenous NF1 GAP activity. Indeed, Yunoue et al. (Yunoue et al., 2003) recently reported that both the R1276K and the R1276A/R1391K constructs significantly reduce the NF1 GAP activity, as well as the Ras and MAPK activity when transfected into PC12 cells and cultured primary hippocampal neurons. Interestingly, these transfected cells also displayed reduced neurite outgrowth, suggesting that NF1 may play an essential role in early neurite outgrowth. Dr. Ming-Xiang Zhang, a Postdoctoral Fellow in the lab has made both mutants from myc-tagged wild-type human pcDNA3.1 NF1GRDI (a gift from Dr. David Guntamann, Washington Uni.). We are now assessing their effects on Ras and MAPK activity using standard cell lines and cultured neurons. If we see a strong effect of these dominant negative constructs, we will proceed to assess their effects on dendritic spine morphology (Yunoue et al., 2003).

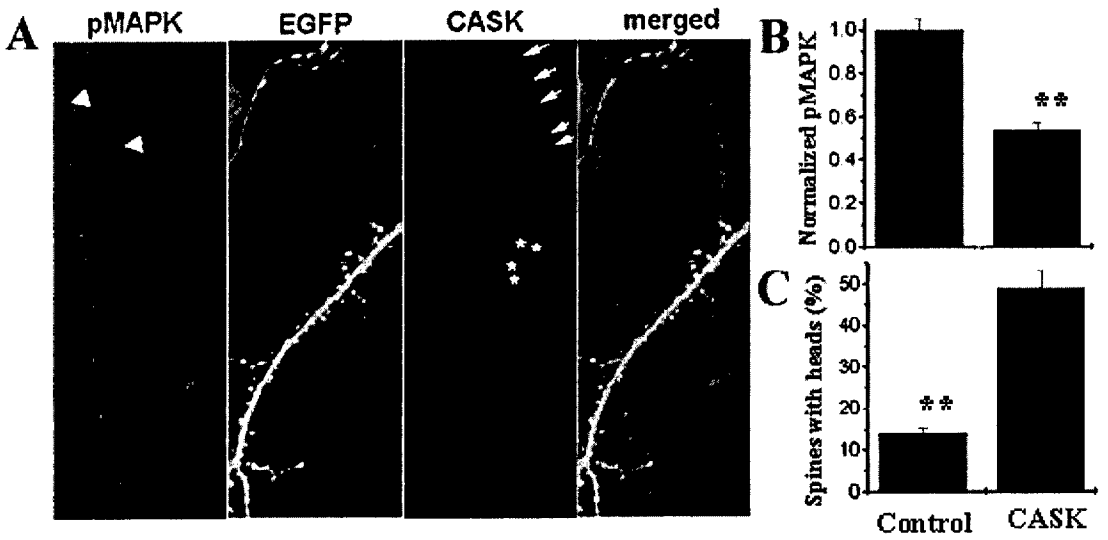

Fig.3. **Overexpression of CASK in young neurons promotes spine maturation and inhibits the MAPK activation.** A. Hippocampal neurons were transfected at 6 DIV. At 8DIV the cells were stimulated by removal of Mg2+ blockade of NMDA nreceptors for 15 min, fixed and stained for pMAPK and CASK. Arrows indicate clustering of CASK in axons, stars indicate clustering of CASK in dendritic spines. Arrowheads indicate the soma of two CASK expressing neurons. B, C. Pooled quantification of overexpression of CASK on the pMAPK (B) and dendritic spine morphology. Values are mean+/- SEM, ** $p<0.01$, $n>20$.

According to our working model, during synaptogenesis, ephrins activate EphB2, which in turn leads to clustering of NMDA receptors and phosphorylation of Syndecan2. The phosphorylated Syndecan2 clusters at synaptic sites and further recruits CASK and NF1. The recruitment of NF1 may function to stabilize the functional spine and limit further morphological plasticity by shutting off the Ras-MAPK signaling. One simple prediction of this model is that overexpression of CASK or Syndecan2 should promote spine maturation as well as down-regulation of pMARK activity during early developmental stages. Vikas Kumar, a graduate student in the lab, has carried out the overexpression experiments, and demonstrated, for the first time that overexpression of CASK in immature hippocampal neurons (8-10 DIV) promotes maturation of both the presynaptic boutons and postsynaptic dendritic spines as evidenced by increasing the numbers of the axonal varicosities (presumed synaptic boutons) and mushroom-shaped spines. In addition, he has been able to confirm Ethell and Yamaguchi's finding (Ethell et al., 2001) that Syndecan2 specifically promotes the maturation of dendritic spines. Remarkably CASK significantly inhibits the pMAPK activity induced by NMDA receptor activation. As

shown in Fig.3, a 5 fold increase (5.1+/- 0.42, n=21) in CASK caused a significant reduction of MAPK activity after removal of Mg^{2+} blockade of the NMDA receptors for 10 min.

To monitor the spatiotemporal activation of Ras in real time, we have made use of a fluorescence resonance energy transfer (FRET)-based probe for Ras. We have finished installation a Zeiss LSM510 META spectral scan confocal microscope and made some improvement in detecting Ras activity using FRET probe. As the original FRET probe has a number of pitfalls such as a small signal/noise ratio and sensitivity to pH and chloride. Ming-Xiang Zhang has successfully replaced the YFP V68L/Q69K with Venus, a new variant of YFP with fast and efficient maturation and reduced pH and chloride sensitivity. We have been able to photobleach a small group of spines to detect the FRET signals at different time point after patterned stimulation. We expect to make good progress in the methodology for detecting the FRET signal in real time by calculating the CFP/YFP emission ratio with correction of the signal bleed-through and pH effects in the next several months. After refining the methodology, we will proceed to directly assess the spatiotemporal regulation of Ras activity in wild type and Nf1 deficient neurons. We expect to complete this line of study by the end of the second year. As a complementary approach, we have been able to use GST pull-down assays showing that Nf1+/- hippocampal tissue display hyperactive basal Ras activity (data not shown).

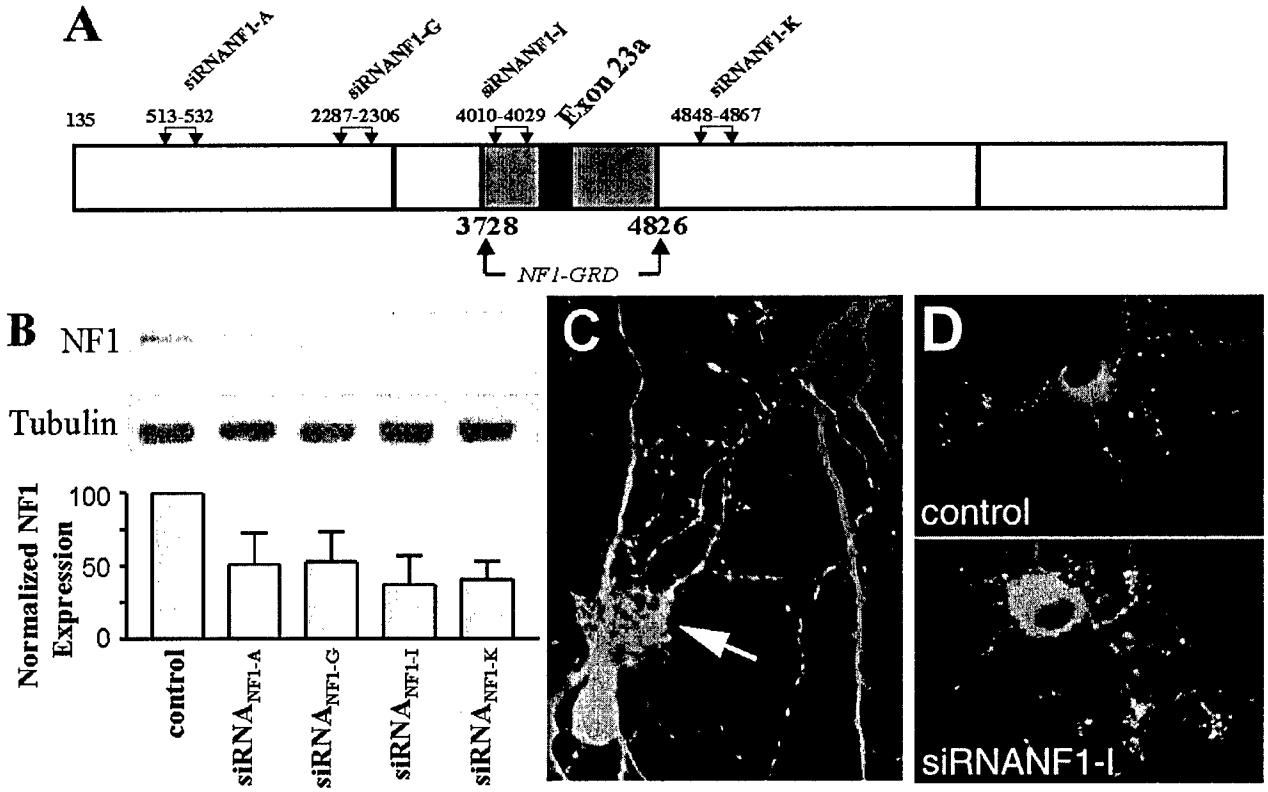

Fig.4. **Use of siRNAs to Knockdown NF1.** (A) Schematic drawing shows the structure of NF1 and the target positions of some of the siRNAs. (B) Western blot shows effective supression of the NF1 by 4 different siRNAs for NF1. Note that typically ~ 60% cells were transfected in our Hela cell cultures; therefore, the actual inhibition may be larger than the values showed here. As one of the controls, the siRNA for EGFP has no effect (not shown). The same Western blots re-probed for other endogenous or overexpression of non-related proteins (such as CaMKII) showed no significant non-specific reduction of these proteins (not shown). (C) siRNAs for NF1 induced immature dendritic phenotypes. The DG explant was first transfected with DsRed along with a control vector at 6 DIV (red cell). Two days later, the explant was transfected with GFP along with $siRNA_{NF1-A}$. The explant was then fixed and imaged at 17DIV. Arrow, cluster of immature filopodia reminiscence of those seen in Ras+ expressing cells (Fig.5C). Similar immature spine phenotypes were seen in cells expressing other siRNAs for NF1 (not shown). (D) Immature spine phenotype revealed by expressing actin-GFP. Note the long filopodia and more diffuse actin-GFP signals in the cell transfected with $siRNA_{NF1-I}$ (imaged at 17DIV and tranfected at 9DIV).

2). Task 2: To assess the role of the NF1-syndecan-CASK signaling complex in regulation of dendritic spine morphology

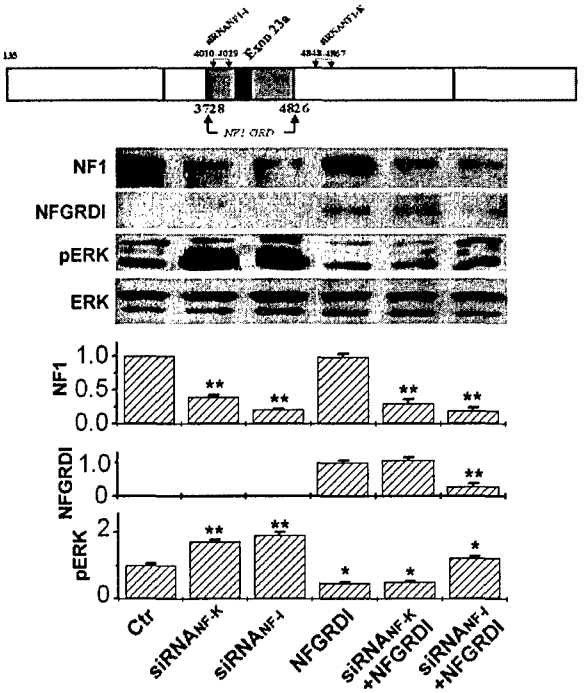

Fig.5 **Specificity of NF1 Knockdown and hyperactive pMAPK.**
Hela cells were co-transfected with different constructs for 48hrs and assayed for immunoblotting, as detailed in Fig.5. Note that overexpression of NF1GRDI, a central domain of NF1 about ~360 residues responsible for its Ras GAP activity, was able to largely rescue the pMAPK level in cells co-expressing the $siRNA_{NF1\text{-}K}$ with NF1GRDI. Cells co-expressing the $siRNA_{NF1\text{-}i}$ with NF1GRDI still showed elevated hyperactive pMAPK. The slightly reduced pMAPK activity was due to incomplete knockdown of NF1GRDI by $siRNA_{INF1\text{-}i}$. * $p<0.05$, ** $p<0.01$; n=3; one-way ANOVA.

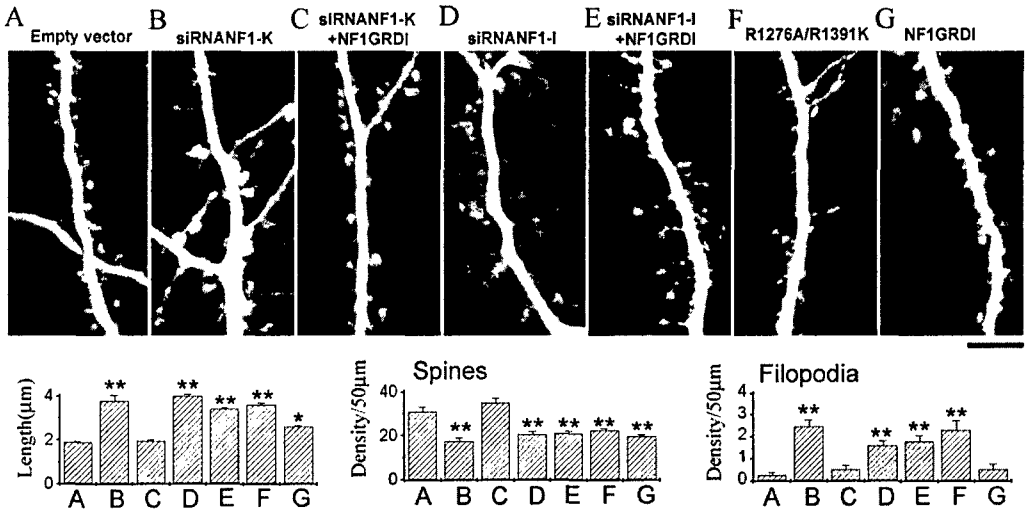

Fig.6 **NF1 siRNAs produced immature spine phenotypes with prominent filopodia or loss of dendritic spines.** DG explants were co-transfected EGFP with different constructs at 7 DIV and imaged at 17DIV. The upper panels show typical spine morphology in the respective groups. The lower panels show quantification for spine and filopodium density, and spine length. Value are means +/-SEM, * $p<0.05$; ** $p<0.01$, one-way ANOVA. Note that co-expression of $siRNA_{NF1\text{-}K}$ with NF1GRDI but not co-expression not co-expression of $siRNA_{NF1\text{-}i}$ with NF1GRDI largely rescued the immature spine phenotype. The dominant negative R1276A/R1391K for NF1 GAP also produced a similar immature spine phenotype. Data were from two independent experiments. N = (A: 715 spines, 18 neurons); (B: 599, 28); (C: 353, 11); (D: 319, 32); (E: 424, 25); (F: 501, 30); (G: 472, 19). Scale bar, 10μm.

Our preliminary results in rat hippocampal cultures showed that overexpression of Syndecan2 promoted the maturation of dendritic spines; overexpression of CASK, on the other hand, seemed to promote the maturation of both the presynaptic boutons and dendritic spines in immature neurons. We have continued making excellent progress showing that knockdown of each of the three proteins using a vector-based RNA mediated interference (RNAi) method produced a similar immature spine phenotype with prominent filopodia or loss of dendritic spines in mature neurons (Figs 4-6). Our latest pilot experiments showed that co-expression of $siRNAI_{NF1\text{-}K}$ with NF1GRDI but not co-expression of $siRNA_{INF1\text{-}i}$ with NF1GRDI largely rescued the immature spine phenotype (Fig.6), strongly supporting that these observed immature phenotypes indeed are due to loss of NF1 expression.

3). Task3: To determine if NF1-deficient cells or NF1 deficient mice have an altered capacity to undergo morphological plasticity after spaced depolarizing stimuli, and if deficits in morphology can be rescued by manipulating Ras-MAPK signaling.

This aim will complement the studies in Aim1 &2 on the role of NF1 in development, and determine if NF1 also plays an essential role in dendritic spine plasticity. If so, additional pharmacological interventions and genetic manipulations of the Ras-MAPK pathway will be used to rescue the morphological deficits. A similar combination of the confocal time-lapse imaging of morphological changes with the assessment of spatiotemporal activation of the Ras-MAPK pathway will further link the morphological plasticity to changes in signal transduction pathways in this activity-dependent model. We have not yet started this series of experiments and will begin at 18 months as planned.

III. Key Research Accomplishments:

- Immunoblot analysis and immunohistochemistry demonstrated that Nf1+/- neurons display hyperactive basal and evoked MAPK activity.

- Developed several siRNAs that target regions either in or outside of the NF1GRDI to specifically knockdown NF1 expression.

- Conducted pilot experiments with the siRNAs showing that knockdown of NF1 produced hyperactive Ras-MAPK signaling and immature spine phenotype

- Refined the FRET probe for Ras and used GST pull-down assay to confirm Nf1+/- neurons display hyperactive basal Ras activity

- Developed two dominant negative constructs for NF1 GAP activity and their effects on Ras-MAPK activity and spine morphology are being tested.

- Demonstrated that overexpression of CASK promoted the maturation of both the presynaptic boutons and dendritic spines with concomitant reduction of MAPK activity in immature neurons

IV. Reportable outcomes:

1). Papers

1. Kumar, V., Zhang, M.X., Kunz, J. and Wu, G-Y. A Central Role of the Ras- PI3K-AKT-mTOR Signaling Pathway in Dendrite Morphogenesis, Neuron, in revision.

2). Presentations: Annual Neuroscience Meeting, San Diego, 2004

1. Zhang, M.X., C.J. Hong, Kumar V. Hsueh, Y.P. and Wu, G.Y. NF1 Signaling and Dendritic Spine Morphogenesis. Soc. Neurosci. Abstr. 2004, 388.14.

3). Other publications during this period

1. Varga, A.W., Yuan, L.L., Anderson, A.E., Schrader, L., Wu, G-Y, Johnston, D. and Sweatt, J.D. (2004) Calcium-calmodulin-dependent kinase II modulates Kv4.2 channel expression and upregulates neuronal A-type potassium currents, J. Neurosci, 24:3663-54.
2. Tang, W., Ingalls CP, Durham WJ, Snider, J., Reid, M.B., Wu, G-Y, Matzuk, M.M and Hamilton,S.L. (2004) Altered excitation-contraction coupling with skeletal muscle specific FKBP12 deficiency, FASEB J., 18:1597-99.
3. Bryan, B., Kumar, V., Stafford, L.J., Xia C., Cai, Y., Wu, G.Y. and Liu, M. (2004) Modulation of Neurite Outgrowth and Spine Formation by GEFT, A Guanine Nucleotide Exchange Factor for the Rho Family of Small GTPases, JBC, 279:45824-32.
4. Bryan B., Cai Y., Wrighton K, Wu G-Y, Feng XH and Liu M. (2005) Ubiquitination of RhoA by Smurf1 promotes neurite outgrowth, FEBS Lett. 579:1015-9.
5. Ryan, X.P., Alldritt J.L., Wu, G-Y, Allen, P., Nairn, A.C. and Greengard, P. Interaction of the Rho GEF, LFC, with neurabin and spinophilin: A link between the microtubule and actin cytoskeletons in dendritic spines, Neuron, in revision.
6. Kumar, V., Zhang, M.X., Kunz, J. and Wu, G-Y. A Central Role of the Ras- PI3K-AKT-mTOR Signaling Pathway in Dendrite Morphogenesis, Neuron, in revision.
7. Maria V. Tejada-Simon, M.V., Serrano, F., Villasana, L.E., Kanterewicz, B.I., Wu, G-Y, Quinn M. and Klann, E., Synaptic localization of a functional NADPH oxidase in the mouse hippocampus, MCN, in press.
8. Wu, G.Y. and Wang, S.R. Telecephalic Striatum Exerts Inhibitory Action on Binocular Neurons in the Toad's Tegmentum, submitted to Neuroscience. Letters.
9. Alldritt, J.L., Ryan, X.P., Allen, P., Nairn, A.C. Greengard, P. and Wu, G-Y, Lfc Regulates Dendrite Formation through Rho-ROCK signaling pathway, in preparation.
10. Kumar, V., Zhang, M.X., Cao Y-Q, Chen G., Tsien, R.W. and Wu, G-Y, Rapidly reversible dendritic swelling is correlated with protein mobilization and formation of dendritic protrusions, in preparation.
11. Zhang, M.X., Kumar, V., Chen, G., Cao Y-Q, Deisseroth, K., Tsien, R. W. and Wu, G-Y, Expression of an active Ras alters the maturation of dendritic spines, in preparation.

V. Conclusions

In summary, the NFRP fund has provided us the crucial support for our research on the biological function of the newly identified NF1-syndecan-CASK signaling complex. With the support, we have been able to make significant progress on several innovative approaches to manipulate the signaling complex and image the underlying signal transduction mechanisms at single cell level. We will continue the proposed study as planned in the original proposal during the second year of the NFRP support, in particular focusing on EphB regulated Ras-MAPK signaling and spine morphology. The combination of structural and functional analyses with the assessment of the underlying signal transduction mechanisms at the single cell level, should provide better new insights into NF1 function in neurons and will shed light on the mechanism by which dysregulation of this function leads to cognitive deficits in NF1 patients. Data obtained from this study will provide critical information needed to develop therapeutic strategies to target the cognitive deficits in NF1.

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VII. Appendices: None